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Inventor(s): BERTRAND BERREBI ISABELLE; BRIL ANTOINE MICHEL ALAIN
Applicant(s): SMITHKLINE BEECHAM LAB (FR)
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Abstract

A process for detecting modulators of myocardial relaxation, which process comprises assessing a modulating effect of a putative modulator upon the phosphorylation of phospholamban and immobilised SR vesicles for use in such process.

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(71) Applicant *(for all designated States except US)*: SMITHKLINE BEECHAM LABORATOIRES PHARMACEUTIQUES [FR/FR]; 6, esplanade Charles de Gaulle, F–92731 Nanterre Cedex (FR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BERREBI–BERTRAND, Isabelle [FR/FR]; SmithKline Beecham Laboratoires Pharmaceutiques, Z.I. de la Peynnière, Boîte postale 2, F–53101 Mayenne Cedex (FR). BRIL, Antoine, Michel, Alain [FR/FR]; SmithKline Beecham Laboratoires Pharmaceutiques, Z.I. de la Peynnière, Boîte postale 2, F–53101 Mayenne Cedex (FR).

(74) Agent: RUTTER, Keith; SmithKline Beecham, Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).

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A process for detecting modulators of myocardial relaxation, which process comprises assessing a modulating effect of a putative modulator upon the phosphorylation of phospholamban and immobilised SR vesicles for use in such process.

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PROCESS FOR DETECTING MODULATORS OF MYOCARDIAL RELAXATION

The invention relates to a novel process, in particular to a screening process for identifying compounds having potentially useful medical utility.

At the cellular and molecular levels myocardial relaxation primarily depends upon the function of a specific enzyme located in the sarcoplasmic reticulum (SR) - SR-Ca^{2+} ATPase. This enzyme mediates the uptake of Ca^{2+} ions into the SR via a cardiac Ca^{2+} pump.

This pump is known to be regulated by a particular phosphoprotein, phospholamban (PLB). PLB is a known regulatory subunit present in two forms - phosphorylated (PPLB) and the aforementioned dephosphorylated form. The dephosphorylated form acts to suppress the activity of the Ca^{2+}ATPase by decreasing the affinity of the pump for Ca^{2+}. This inhibitory effect is relieved - thereby promoting cardiac relaxation - when PLB is phosphorylated to PPLB.

The phosphorylation of PLB is catalysed by a protein kinase (PK). The dephosphorylation of PPLB is catalysed by a particular protein phosphatase (PP) PLB-PP.

We are interested in studying the conversion of PPLB to PLB and in particular in the detection of compounds which inhibit the activity of PLB-PP.

The PLB-PP associated with cardiac SR is a type 1 enzyme or PP1. The catalytic activity of PP1 is known to be controlled by a 37 kiloDalton (kDa) protein subunit, PP1Csub.

Recent evidence indicates that a novel class of proteins, of 124 kDa, named targeting subunits (PP1-Tsub), is functionally linked to PP1Csub and ensures that the binding of PP1Csub to SR takes place at a specific subcellular location (Tloc) on SR. In the heart, the PP1Csub+PP1-Tsub heterodimer is called PP1G and is the major SR-PP.

It is our view that the binding of the PP1-Tsub complex which occurs with PLB in the SR provides a signal intervention point into the process of formation of PPLB and hence for modulating Ca^{2+}-ATPase activity. A corollary to this is that substances which modulate this binding and thereby modulate the suppression of Ca^{2+}-ATPase activity would potentially have a beneficial effect upon myocardial relaxation.

Accordingly, in a first aspect the present invention provides a process for detecting modulators of myocardial relaxation, which process comprises assessing a modulating effect of a putative modulator upon the phosphorylation of phospholamban.

Suitable modulators include proteins and small molecules, especially small molecules.

Suitable modulators are inhibitors or antagonists of the dephosphorylation of phospholamban .

Inhibitors or antagonists of the dephosphorylation of phospholamban are potentially useful for the treatment of cardiac disorders associated with an inhibition of myocardial relaxation, such as congestive heart failure, left ventricular hypertrophy and cardiac arrhythmias.

The invention is considered to extend to modulators, in particular the inhibitors or antagonists, identified by use of the process of the invention.

Suitable assessments of the modulating effects comprise detecting and/or characterising the said modulating effects.

In a preferred aspect, the dephosphorylation of phospholamban is assessed via an interaction, suitably a binding interaction, between phospholamban and a protein phosphatase (PP), suitably PP1Csub and especially PP1Csub-PP1-Tsub complex.

The phospholamban is suitably located in sarcoplasmic reticulum vesicles.

A suitable source of PP1-Tsub has been obtained from an homosapiens PPP1R3 mRNA for protein phosphatase1. The Genbank accession number is X78578. The PP1-Tsub clone was obtained already inserted in BlueScript plasmid. The sequence of the human glycogen-associated regulatory subunit of type 1 protein phosphatase has been published in Diabetes 43, (10) 1234-1241 (1994).

PP1Csub and anti PP1Csub are both commercially available: for example both can be obtained from Euromedex [PP1Csub, ref 14-110 and anti human PP1Csub, ref 06-22].

PLB has been produced in rabbit reticulocyte lysate using an "in vitro" transcription/translation system (Promega). This methodology offers several advantages including rapid protein synthesis and ^{35}S Methionine labelling. The products can be rapidly analysed by SDS PAGE using autoradiography and immunoblot experiments.

Labelled PLB protein has been successfully produced in small amount by this technique: PLB protein appears to be produced in its multimeric form in rabbit reticulocyte lysate and migrated slowly. When lysate denatured, the PLB protein co-migrated with the control form purified from dog RS (about 25 Kd).

Any conventional methodology can be employed to assess the interaction between PLB and PP1-Tsub, for example biosensor technology, radiobinding method, scintillation proximity assays, co-immunoprecipitation assays, native page electrophoresis or cross linking experiments methods.

In one particular form of the process, radiolabelled 35S-PP1-Tsub is used, the effect of a putative modulator was then assessed by determining the effect of

the putative modulator upon the transfer of the label to PLB using conventional radioisotopic methods.

One particularly advantageous aspect of the process is provided by the immobilised SR vesicles, which may be solubilized or non-solubilised.

Surprisingly, the PLB of the immobilised vesicles retained binding capacity as demonstrated by interaction with PLB monclonal antibodies. In the same way polyclonal antibodies raised against PP1-Tsub are able to bind to SR vesicles, suggesting the presence of viable PP1-Tsub in the immobilised preparations.

Accordingly, immobilised SR vesicles, for example dog vesicles, form a further part of this invention, preferably using aminosilane coated micro-cuvettes. Micro-cuvettes are available with a choice of derivatised sensor surfaces, including a carboxymethyl-dextran hydrogel, or an aminosilane surface linker specifically developed for large molecules and cells.

Preferably, a binding agent, such as bis (sulfosuccinimidyl) suberate (BS3, 1mM) is used to cross link the vesicles to the immobilisation agent surface.

The vesicles are immobilised using known technology, for example that described below.

The monoclonal PLB antibodies are obtained from Upstate Biotechnology (ref 05-205 according to Suzuki and Wang, J Biol.chem 261:7018 1986).

In an analogous fashion the pharmacological viability of the PP1-Tsub may be validated by demonstrating an interaction between the PP1-Tsub and polyclonal antibodies raised against active PP1-Tsub

The process of the invention is illustrated by the following Example.

Example: Protein-Protein Interaction: Assay using Biosensor Technology.

a) The protocol (FISONS Applied Sensor Technology) for immobilising the vesicles is the following:

1) Activation: A baseline is established with sodium phosphate buffer which is then replaced with 200 ul of BS3 (Pierce, 21579) solution for 10 minutes, followed by aspiration and washing thoroughly with buffer.

2) Immobilization: Once a baseline was achieved 100 µg/ml SR was added to the vesicles for 10 minutes which were then washed with buffer.

3) Blocking: After immobilisation, in order to block any remaining sites on the activated surface, 200 ul of bovine serum albumin (BSA, Sigma A-6003) was added to the cuvette for 5 minutes followed by aspiration and washing thoroughly with buffer.

4) The sodium phosphate buffer was then changed to a suitable buffer for the binding stage, eg PBS.

5) The SR vesicles are then added in the binding experiments.

6) Regeneration procedures are then used to allow rapid and frequent re-use of the immobilized ligand.

b) Assay Procedure:

Dog SR Vesicles were solubilised with 1% Zwittergent in a cuvette and successfully immobilized onto aminosilane surface.
Monoclonal antibody raised against PLB was then added. This evidenced an interaction with solubilised SR vesicles demonstrating that the PLB is still pharmacologically accessible after the immobilization. Binding and dissociation are increasingly important measures of biological activity and function. Affinity sensors is an optical biosensor system for studying biomolecular interactions in real-time. It allows reactions to be watched as they happen, so revealing the dynamics as well as the strength of binding. Analysis is carried out very rapidly without the need for labels. Binding and dissociation are seen as shifts in resonance angle arising as one partner or more in free solution binds to, or dissociate from, the other partner immobilized at the surface of the sensor.

In the same way anti-PP1-Tsub is able to bind to solubilised SR vesicles suggesting the presence of PP1-Tsub in our preparations

As a negative control, SR vesicles isolated from skeletal muscle were shown to give no reaction with the PLB monoclonal antibodies as there is no PLB in skeletal muscle.

When monoclonal antibodies raised against PLB were immobilized on aminosilane surface, PLB is able to recognize anti-PLB antibodies. The addition

of PP1-Tsub at this stage clearly demonstrate for the first time the interaction between PP1-Tsub and PLB.

Results and Conclusion

The results obtained with Phospholamban, PP1-Tsub, solubilised dog cardiac vesicles are shown in the table and clearly illustrate that the interaction exists between phospholamban and PP1-Tsub (Tab).

	SR solubilised vesicles	Anti-PLB	Anti-PP1-Tsub
PLB	++	++	--
PP1-Tsub	+++	--	++
Cardiac SR vesicles		+++	++
Skeletal muscle SR vesicles		--	+++

-- no interaction,
++ interaction
+++ strong interaction and high affinity between the two partners.

The results from the Table are interpreted as follows, referring in turn to the interactions represented by each box of the Table:

- PLB-SR vesicles: binding with a structure in the SR (probably PP1-Tsub)
- PLB-anti-PLB: binding between the protein and the specific antibodies raised against this protein
- PLB-Anti PP1-Tsub:no recognition as expected
- PP1-Tsub-SR vesicles: binding with a structure in the SR (probably PLB)
- PP1-Tsub -anti-PLB: no recognition as expected
- PP1-Tsub -Anti PP1-Tsub: binding between the protein and the specific antibodies raised against this protein

- Cardiac SR vesicles-anti-PLB: binding as PLB present in SR vesicles
- Cardiac SR vesicles-anti-PP1-Tsub: binding suggesting that PP1-Tsub is present in SR vesicles
- Skeletal muscle SR vesicles-anti-PLB: no recognition as PLB not present in skeletal muscle
- Skeletal muscle SR vesicles-anti-PP1-Tsub: binding as PP1-Tsub is also expressed in skeletal muscle.

Conclusion:

This assay shows that the interaction between PLB and PP1-Tsub represents a useful target for drug action aimed at the treatment of the above mentioned cardiovascular diseases.

Notes:

Dog SR vesicles were prepared according to Jones et al., J. Biol. Chem 1979,254,530-539.

Monoclonal antibodies raised in mouse against PLB are obtained from Upstate Biotechnology (ref 05-205 according to Suzuki and Wang, J Biol Chem 261:7018 1986).

Polyclonal antibodies raised in sheep against PP1-Tsub were obtained from Philip Cohen (University of Dundee).

Claims:

1. A process for detecting modulators of myocardial relaxation, which process comprises assessing a modulating effect of a putative modulator upon the phosphorylation of phospholamban.

2. A process according to claim 1, wherein the dephosphorylation of phospholamban is assessed via an interaction, between phospholamban and a protein phosphatase (PP)

3. A process according to claim 1 or claim 2, wherein the interaction, between phospholamban and a protein phosphatase (PP) is a binding interaction.

4. A process according to any one of claims 1 to 3, wherein the protein phosphate is located in sarcoplasmic reticulum vesicles.

5. A process according to any one of claims 1 to 4, wherein the protein phosphate is PP1Csub-PP1-Tsub complex.

6. A process according to claim 5, wherein PP1-Tsub is obtained from an homosapiens PPP1R3 mRNA for protein phosphatase1.

7. A process according to claim 5 or claim 6, wherein PP1-Tsub is radiolabelled as 35S-PP1-Tsub.

8. Immobilised SR vesicles.

9. Immobilised SR dog vesicles.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/42 G01N33/68 C12N5/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	I. EDES & E. G. KRANIAS: "Review: Regulation of cardiac sarcoplasmic reticulum function by phospholamban" MEMBRANE BIOCHEMISTRY, vol. 7, no. 3, 1987 - 1988, pages 175-192, XP002053752 see the whole document	1-4
A	---	8,9
X	K. TAKISHIMA ET AL.: "A spin-label study of the effects of drugs on calcium release from isolated sarcoplasmic reticulum vesicles." JOURNAL OF BIOCHEMISTRY, vol. 87, no. 1, 1980, TOKYO, JP, pages 305-312, XP002053753 see the whole document	1-4

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[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex.

' Special categories of cited documents

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Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 19754 A (SMITHKLINE BEECHAM PLC ;SMITHKLINE BEECHAM LAB (FR); MURRAY KENNET) 14 October 1993 see the whole document	1
P,X	K. L. KOSS & E. G. KRANIAS: "Phospholamban: a prominent regulator of myocardial contractibility" CIRCULATION RESEARCH, vol. 79, no. 6, December 1996, pages 1059-1063, XP002053754 see the whole document	1-4
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ›nal Application No
PCT/EP 97/05265

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